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Genome-wide exploration identifies sex-specific genetic effects of alleles upstream NPY to increase the risk of severe periodontitis in men

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Abstract

Aim: Periodontitis (PD) is influenced by genetic as well as lifestyle and socio-economic factors. Epidemiological studies show that men are at greater risk of severe forms of PD, suggesting interplay between sex and genetic factors. We aimed to systematically analyse patients with aggressive periodontitis (AgP) for gene–sex interactions.

Materials and Methods: Three hundred and twenty-nine German AgP cases and 983 controls were genotyped with Affymetrix 500K Arrays and were analysed by logistic regression analysis. The most significant gene–sex interaction was replicated in an independent sample of 382 German/Austrian AgP cases and 489 controls.

Results: Ten single-nucleotide polymorphisms (SNPs) in strong linkage disequilibrium ($r^2 > 0.85$) upstream the gene neuropeptide Y (*NPY*) suggested gene–sex interaction ($p < 5 \times 10^{-5}$). SNP rs198712 showed the strongest association in interaction with sex ($p = 5.4 \times 10^{-6}$) with odds ratios in males and females of 1.63 and 0.69 respectively. In the replication, interaction of sex with rs198712 was verified with $p = 0.022$ (pooled $p = 4.03 \times 10^{-6}$) and similar genetic effects. Analysis of chromatin elements from ENCODE data revealed tissue-specific transcription at the associated non-coding region.

Conclusion: This study is the first to observe a sexually dimorphic role of alleles at *NPY* in humans and support previous genome-wide findings of a role of *NPY* in severe PD.

Key words: genetic association; interaction; *NPY*; Periodontitis; sex

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Periodontitis (PD) is a chronic inflammatory disease of the oral cavity. The inflammation is elicited by the oral microbial biofilm that leads to gingival bleeding, pocket formation, attachment and bone loss and eventually to tooth loss as final outcome (Buchwald et al. 2013). PD affects human populations worldwide at prevalence rates of 11% for the severe forms (Marcenes et al. 2013). It is largely classified into the sub-forms chronic periodontitis (CP) and aggressive periodontitis (AgP). Whereas CP is mostly observed in adults and is characterized by slow progression of the disease, AgP is found in young individuals and is diagnosed based on rapid attachment loss and destruction of the alveolar bone. A pathogenic microflora is the causing agent of PD; however, factors such as smoking, diabetes and obesity can contribute to the disease risk (Mealey & Oates 2006, Buchwald et al. 2013, Sangwan et al. 2013, Schenkein & Loos 2013). Related to these risks, socio-economic factors such as poor educational attachment and low income are also associated with the progression of PD and tooth loss (Buchwald et al. 2013). For CP, epidemiologic studies provide broad-based evidence that men are at greater risk of developing severe periodontal disease than women (McGrath & Bedi 2003, Shiau & Reynolds 2010). A recent study estimated the prevalence, severity and extent of periodontitis in the adult US population with data from the 2009 and 2010 National Health and Nutrition Examination Survey (NHANES) cycle and reported that after adjustment for the effect of age, total periodontitis was significantly higher in men than in women aged 30 years and older (males = 57%; females = 39%), with males showing a 3%, 7% and 8% higher prevalence of mild, moderate and severe PD, respectively, compared with women (Eke et al. 2012). Findings like these suggest interplay between genetics, sex and possibly other environmental factors in PD. For AgP, an increased prevalence in males or females is currently not supported in the literature (Hart et al. 1991). However, the few available studies are small, which decreased their ability for generalization.

A genetic basis of PD has been known by formal genetic studies for many years (Michalowicz et al. 1991, 2000, Corey et al. 1993, Marazita et al. 1994) but compared to other complex diseases a relatively low number of risk alleles have been identified that provide evidence of association by repeated replication in independent, large case–control populations (Schaefer et al. 2013).

In common with other complex diseases, there is a gap between statistical modelling and biological phenomena, which is notoriously understudied. In general, genetic susceptibility regions were analysed for the average proband, but further subject characteristics such as sex were not taken into account. A limitation of this approach is that relevant influential factors can be overlooked simply because they exert their effect only against specific patterns of expression of other factors.

We hypothesized interactions between specific SNPs and sex that influence the disease risk of PD. To test this hypothesis, the genetic constitution and the interaction term of sex were analysed on a genome-wide level in a case–control sample of the early-onset phenotype AgP. A focus on this phenotype allowed a better control of confounding effects such as age and accumulating lifestyle factors and due to the strong severity at a young age, it is assumed that genetic factors play a prominent role in disease susceptibility. The main result was verified in an independent AgP case–control replication sample.

Material and Methods

Study population

Cases and controls that were genotyped with Affymetrix 500K Arrays were described before (Schaefer et al. 2010). In brief, inclusion criteria for the 329 German AgP cases (131 males, 189 females) of the GWAS panel were ≥2 teeth with 50% alveolar bone loss from the cemento-enamel junction to the tooth apex in subjects who were under the age of 35 years and whose parents and grandparents were born in Germany. To limit a potential bias in the diagnosis of the disease phenotype by different examiners, a set of full-mouth dental radiographs was used. Copies were mailed for independent confirmative periodontal bone scoring to the study centre and the initial diagnosis was confirmed by a second specialist in periodontology who has, without exception, been located and educated at the study centre. The use of radiographs allowed a reproducible and quantifiable diagnosis of the disease phenotype. The controls were population representative individuals from the region of Kiel, Germany (N = 500) and blood donors from the University-Clinic Schleswig-Holstein, Kiel, Germany (N = 500) collected by the biobank popgen (Krawczak et al. 2006). The inclusion criteria for the 382 German/Austrian cases of the replication panel (174 males, 208 females) were ≥2 teeth with 30% bone loss under the age of 35. The parents and grandparents of the German cases were born in Germany. The parents and grandparents of the Austrian cases (N = 69) were born in Austria with German family names. These cases were described in Schaefer et al. (2013). The controls of the German replication sample were 489 population representative controls from South-Germany (246 males, 243 females), provided by the Cooperative Health Research in the Region of

Augsburg Study (KORA), Bavaria, Germany (Wichmann et al. 2005). Population representative controls and blood donors were generally regarded to be free of AgP, as the prevalence of AgP is very low with an estimated occurrence of <0.1%.

The study was approved by each institute's own ethical review board and all participants provided written informed consent.

DNA isolation and genotyping

Genomic DNA was extracted from frozen blood samples. All DNA samples were quality controlled on agarose gels. Genotyping of the GWAS was performed with the Affymetrix Gene Chip Human Mapping 500K Array Set for patients and controls (Schaefer et al. 2010). Genotypes were assigned using the BRLMM-p algorithm. In the replication, SNP rs198712 was genotyped with the TaqMan Assay hCV9946741 (Applied Biosystems, Foster City, CA, USA) on an automated platform, employing TECAN Freedom EVO and 96-well and 384-well TEMO liquid handling robots (TECAN, Männedorf, Switzerland).

Statistical analysis

SNPs with a genotype call rate <90% and a MAF >5% were excluded from the study. Markers were tested for deviations from Hardy–Weinberg equilibrium in controls before inclusion into the analyses ($\alpha = 0.05$). From a total of 500,568 SNPs, 65% passed the quality criteria (287,224 SNPs). Possible statistical interactions between remaining SNPs and sex on AgP were assessed by logistic regression analysis at a significance threshold of $p < 0.05$. The number of alleles, specified as a continuous influence factor (additive model) and sex were included in the model equation as main effects. From these models, we report p values and sex-specific odds ratios (ORs) that were calculated for the explorative GWAS data, for the replication data and for both data pooled. Model-based multifactor dimensionality reduction (Calle et al. 2010) was performed using the open source software MB-MDR version 4.1 (University of Liège, Belgium) for a binary phenotype, fixing sex as interaction partner for all individual SNPs. Statistical analyses were conducted using PLINK v2.049 (Purcell et al. 2007) and the R software package, version 2.12.2 (http://www.r-project. org).

In silico analysis of chromatin state segmentation

Chromatin state segmentation of human cell lines was produced by ChIP-seq data (Chromatin In situ Precipitation) generated by Broad Institute, (Bernstein laboratory, Massachusetts General Hospital/Harvard Medical School) and produced in Manolis Kellis's Computational Biology group at the Massachusetts Institute of Technology. Chromatin states were learned from this binarized data using a multivariate Hidden Markov Model (HMM) as previously described (Ernst et al. 2011). Data were publicly available by UCSC Genome Browser and were generated by the ENCODE (Encyclopedia of DNA Elements) consortium.

Results

Genome-wide exploration of sex-specific effects

All SNPs that were genotyped on the Affymetrix 500K Array and passed the quality criteria (287,224 SNPs) were analysed in a logistic regression analysis. A total of 2,041 SNPs had a $p < 0.05$ in the main effect of sex, SNP or the gene–sex interaction term. The lowest p-values were observed for an intergenic region on chromosome 7 (chr.7:2414 3041-24149638; NCBI build 36/hg18), spanning 6.597 kilobasepairs (kb; Table S1). This region is located 140-kb upstream the gene neuropeptide Y (*NPY*) (chr7:24,290,334-24,298,002) and was covered by 11 successfully genotyped SNPs with an average distance of 0.44 kb (±0.43 kb). Of these SNPs, 10 showed a p value in the gene–sex interaction term less than 5×10^{-5} (Table 1) and were in high linkage disequilibrium [(LD) $r^2 > 0.85$; Fig. 1a]. The lead SNP was rs198712 with a $p = 5.4 \times 10^{-6}$ for the interaction and $p = 9.8 \times 10^{-6}$ and $p = 0.0240$ for SNP and sex, respectively (Table 1). The sex-specific ORs for the additive effect were 1.629 for males and 0.689 for females. Corroborating this finding, this SNP showed the strongest interaction effect with sex in the genome-wide MB-MDR analysis. The MAF of the male cases and controls was 48% and 36%, and of female cases and controls 30% and 39%, respectively. The MAF in the controls (males and females) as well as in the cases (males and females) was 37% (genotypes are shown in Table 2). Accordingly, in the ordinary association

Table 1. Top 10 results of gene–sex interaction from logistic regression models of GWAS data and in the replication of tag SNP rs198712

SNPs	p SNP	p sex	OR	95% CI	p value	Cases		Controls	
						MAF (males)	MAF (females)	MAF (males)	MAF (females)
rs198733	1.74E-05	0.112	2.17	1.55–3.16	2.02E-05	50.38	34.01	37.11	40.95
rs198731	7.04E-05	0.149	2.07	1.44–2.97	9.02E-05	49.61	34.38	37.25	40.50
rs198728	9.83E-06	0.075	2.21	1.55–3.16	1.26E-05	50.76	34.01	37.11	40.96
rs115063	1.43E-05	0.096	2.17	1.52–3.09	2.06E-05	50.76	34.52	37.23	41.06
rs198727	9.98E-06	0.076	2.21	1.55–3.16	1.27E-05	50.76	34.01	37.13	40.96
rs115062	5.94E-06	0.054	2.27	1.59–3.25	7.09E-06	50.76	33.67	36.91	40.96
rs198720	2.77E-05	0.088	2.18	1.53–3.12	1.80E-05	51.14	35.03	38.90	42.98
rs198712	9.76E-06	0.024	2.36	1.63–3.42	5.41E-06	48.09	29.89	35.73	38.58
rs198711	1.64E-05	0.071	2.20	1.54–3.15	1.64E-05	49.24	32.23	36.35	39.47
rs198701	8.32E-06	0.042	2.27	1.58–3.25	9.45E-06	49.24	32.12	35.83	39.42
Replication: rs198712	0.024	0.2033	1.5	1.07–2.3	0.0222	43.39	38.22	36.99	42.80

tests that considered the cases and controls without taking sex into account, these SNPs showed no significant effect upon AgP.

Replication of the association on chromosome 7 upstream *NPY*

SNP rs198712 was selected for replication in an additional sample of 382 German and Austrian AgP cases and of 489 German controls. In the replication, the p value for interaction was $p = 0.0222$, and the p values for the SNP and sex were $p = 0.024$ and $p = 0.203$ respectively (Table 1). The sex-specific ORs were OR=1.304 for males and OR=0.832 for females, and the MAF was comparable to the values in the initial sample (43% in male cases, 37% in male controls, 38% in female cases, 48% in female controls). After pooling of the explorative and the replication sample, the interaction p value was $p = 4.03 \times 10^{-6}$.

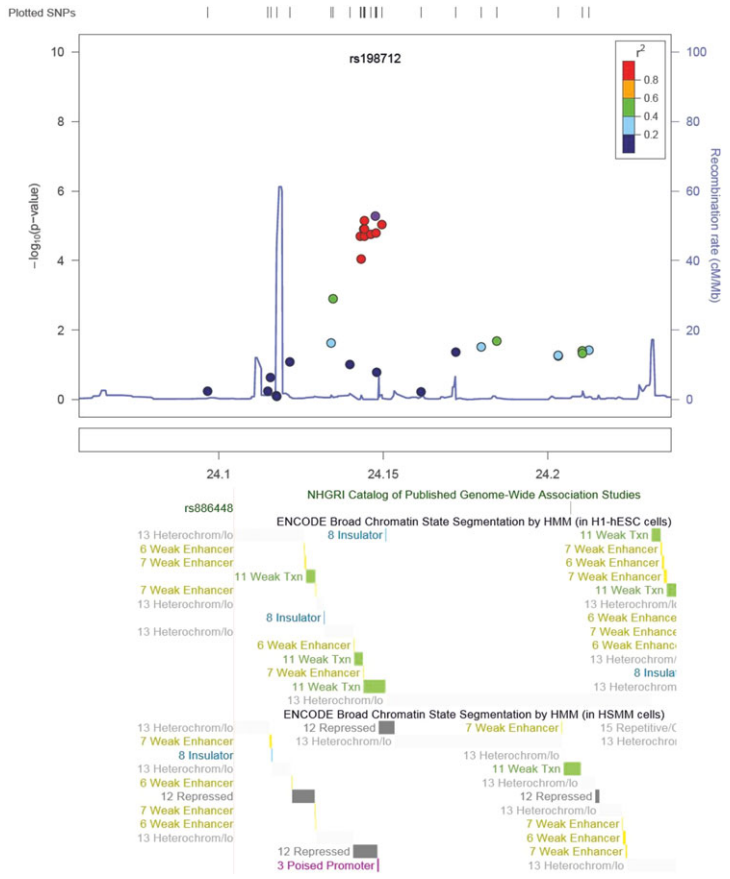

Fig. 1. Chromosomal region at *NPY*. The top diagram shows the nominal $-\log^{10}$ P for the plotted SNPs at this chromosomal region. The $-\log^{10}$ p-values are plotted as a function of the genomic SNP position (NCBI build 36). The second panel shows the position of SNP rs886448, which showed genome-wide association with childhood asthma. The following panel shows annotations of chromatin elements from ENCODE data for two cell types [human skeletal muscle myotubes (HSMM) and H1 human embryonic stem cells (H1-hESC), TXN = transcription]. Tissue-specific transcription (TXN) of the regions, which are associated with AgP and childhood asthma (rs886448) are shown in light green. The plotted SNPs are from left to right: 1 = rs1494194, 2 = rs17287854, 3 = rs2813838, 4 = rs272709, 5 = rs17288003, 6 = rs17148810, 7 = rs272665, 8 = rs272662, 9 = rs1859289, 10 = rs198733, 11 = rs198731, 12 = rs198728, 13 = rs115063, 14 = rs198727, 15 = rs115062, 16 = rs198720, 17 = rs198712, 18 = rs198711, 19 = rs6461782, 20 = rs198701, 21 = rs17290092, 22 = rs156270, 23 = rs156318, 24 = rs156308, 25 = rs156294, 26 = rs156293, 27 = rs13245518, 28 = rs963830, 29 = rs2158342.

In silico analysis of the chromatin state of the *NPY* region

To gain insight into the nature of the associated chromosomal region, we analysed the annotation of chromatin elements of different human cell types from ENCODE data (Yang et al. 2010, Hoffman et al. 2013).

The associated chromosomal region that was tagged by rs198712 showed tissue-specific transcription and possessed a poised promoter (Fig. 1). Further 54-kb downstream within this intergenic region, a genome-wide association with childhood asthma was reported for SNP rs886448 (Ding et al. 2013). This SNP was not in LD with the AgP-associated region ($r^2 = 0$, $D' = 0.58$), but in accordance with this region, tissue-specific transcription was shown for the chromosomal position tagged by the GWAS lead SNP for childhood asthma (Fig. 1). Likewise, at the intergenic region downstream of *NPY* that was reported to be strongly associated with severe CP in a GWAS (rs2521634) and not in LD with the AgP associated region ($r^2 = 0$, $D' = 0.13$) (Divaris et al. 2013), tissue-specific transcription and poised promoters were shown, as was observed for the AgP associated region (Fig. 2). The CP-associated region was also described to confer risk of early-onset atherosclerosis in the Framingham SHARe data, i.e. SNP rs10487606, 40-bp upstream of rs2521634, was associated with increased coronary artery calcium (Shah et al. 2009).

Discussion

In this study, we investigated interactions of SNP genotypes with male and female sex with respect to the risk for aggressive periodontitis on a genome-wide scale. Our main finding was an associated intergenic region 140-kb upstream of the gene *NPY* that conferred an increased risk for AgP in men, but a decreased risk in women.

Following the initial observation of the association of this chromosomal region with AgP, we hypothesized that a genetic region of strong linkage disequilibrium ($r^2 > 0.85$) upstream *NPY* shows gene–sex interaction. This hypothesis was subsequently verified in a second inde-

Table 2. Genotypes of the ten most significant SNPs of gene–sex interaction from logistic regression models of GWAS data and in the replication of tag SNP rs108712

	SNPs	Cases						Controls					
		Males			Females			Males			Females		
		11	12	22	11	12	22	11	12	22	11	12	22
GWAS	rs198733	35	59	36	90	80	27	212	220	80	170	208	86
	rs198731	33	63	32	87	78	27	205	220	77	170	211	82
	rs198728	34	62	36	90	80	27	212	220	80	172	211	87
	rs115063	34	62	36	89	80	28	211	222	80	172	210	88
	rs198727	34	62	36	90	80	27	212	221	80	172	211	87
	rs115062	34	62	36	90	80	26	212	222	78	172	211	87
	rs198720	35	59	38	85	86	26	199	224	86	158	220	92
	rs198712	36	64	31	92	81	16	216	221	71	182	206	76
	rs198711	36	62	34	92	83	22	216	221	76	182	205	83
	rs198701	37	60	35	91	80	22	215	222	71	181	205	82
Replication	rs198712	55	87	32	81	95	32	99	112	35	82	114	47

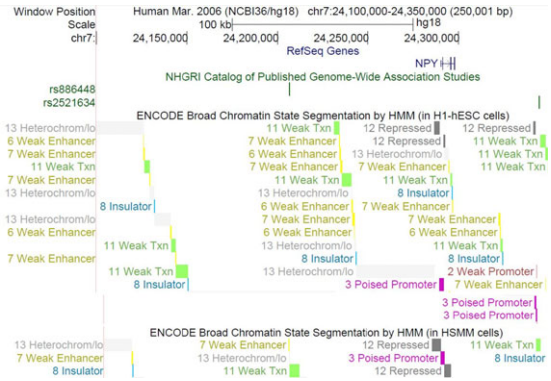

Fig. *2*. Chromosol region at NPY. The top panel shows the chromosomal positions of the covered region. The panels below show the position of NPY (panel 2), the GWAS lead SNPs of childhood asthma and severe CP (panel 3), and of the ENCODE chromatin state segmentations for the two cell types H1-hESC and HSMM (panel 4 and 5), aligned to the chromosomal positions. The covered area includes the chromosomal region of Figure 1 and extends to the GWAS lead SNP of chronic periodontitis (rs2521634).Tissue-specific transcription is shown in light green.

pendent sample. Because all 10 SNPs that showed that the strongest associations in the explorative GWAS analysis were linked on the same haplotype block, rs198712 was replicated as a single tagging SNP. Thus, no correction for multiple testing was required in the replication. In the replication, the association was weaker compared with that observed in the explorative GWAS sample. This could be due to the less severe phenotype of the cases in the replication, with >30% alveolar bone loss at ≥2 teeth compared to >50% bone loss in the cases of the GWAS sample at the same age of disease onset, which can result in a decreased statistical power. Likewise, and corresponding to the higher severity of the cases in the GWAS sample compared with the cases of the replication, the male cases of the GWAS sample had a MAF= 48% (MAF= 36% male controls) compared to a MAF= 43% in the male case group of the replication (MAF= 37% male controls). Alternatively or in addition, the true genetic effect size could be overestimated in the explorative sample as a consequence of the winner's curse phenomenon (Lohmueller et al. 2003).

Interestingly, a recent large GWAS on severe CP described strong association downstream from *NPY* as a main finding (Divaris et al. 2013). Another study that relates *NPY* to CP established the presence of NPY Y1 receptors in the gingival tissue and of NPY protein in human gingival crevicular fluid (GCF), an immunologically relevant exudate of the gingival crevice. This study showed that in the GCF from healthy sites, significantly higher NPY levels were observed compared with periodontitis-affected sites (Lundy et al. 2009). Interestingly, NPY is also the most abundant neuropeptide in bone (Ahmed et al. 1994) and has recently been shown to have a role in maintaining the balance between hard tissue formation and resorption, processes that are relevant to the definition of periodontitis (Haug & Heyeraas 2006). The immunomodulatory effects of NPY are thought to alter the pro-inflammatory T-helper type 1 (Th1): anti-inflammatory T-helper type 2 (Th2) balance, and binding of NPY to Y1 receptors on a variety of immune cells is thought to be responsible for promoting the anti-inflammatory Th2 response. NPY is therefore potentially important in the coordination of inflammation and bone metabolism, both of which are central to the pathogenesis of PD (Lundy et al. 2009).

To our knowledge, this study is the first to observe a sexually dimorphic role of *NPY* in humans that is associated with a complex disease. Interestingly, sex-dependent effects of NPY were previously described in mice. NPY loss-of-function mice showed different anxiogenic responses in behavioural tests in males and females, indicating a sexually dimorphic role of NPY in stress response (Painsipp et al. 2011). Also,

gastrointestinal inflammation, known to enhance anxiety in a sex-dependent manner, produced different behavioural responses to stress challenges in female and male NPY knockout mice (Painsipp et al. 2011). Another study on NPY knockout mice showed sex-dependent responses in food intake, upper gastrointestinal transit and faecal pellet output induced by restrained and novel environment stresses (Forbes et al. 2012).

NPY activates the hypothalamic–pituitary–adrenal (HPA) axis and modulates the visceral stress responses. In addition, NPY is potently anxiolytic (Karl et al. 2008). In accordance with the function in mice, NPY influences many physiological processes in humans, including stress response (Zhou et al. 2008) and stress-induced obesity (Kuo et al. 2007). Often, stress-associated and eating disorders also have a different prevalence among women and men in humans (Kessler et al. 1995, Laughlin et al. 2000). A nonsynonymous SNP (Leu/Pro transition) within *NPY* was associated with serum triglyceride concentrations and birthweight, high serum cholesterol and LDL cholesterol levels (Karvonen et al. 1998), as well as alcohol dependence population samples from the United States (Lappalainen et al. 2002) and Finland (Kauhanen et al. 2000). Stress, obesity and alcohol consumption are considered as risk factors of periodontitis.

In summary, the described findings on the function of NPY add confidence in the validity of our results.

There is a well-known limitation of the logistic regression model in general (Witte & Greenland 1997) as it can describe only multiplicative effects of odds ratios. In this study, it was observed that a specific factor, i.e. sex, masked the genetic effect in a way that the genetic influence could be observed only in sex stratified analyses. Without taking gene–sex interactions into account, this association would have been missed. This showed that adding an interaction term is a first step towards a more comprehensive model attempting to describe more precisely the interplay of environmental and genetic factors.

In conclusion, we identified and replicated the NPY region as conferring an increased risk of AgP in men and a decreased risk in women. Additional studies are warranted to explore the molecular mechanism behind the observed sex-specific effect of genetic variation at *NPY* on the risk of PD.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Association results of gene–sex interaction from logistic regression models of GWAS data with $p < 10E^{-3}$.

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Clinical Relevance

Scientific rationale for the study: Epidemiological studies showed that men are at greater risk of severe forms of periodontitis suggesting interplay between sex and genetic factors.

Principal findings: Ten alleles upstream the gene neuropeptide Y (*NPY*) suggested gene–sex interaction ($p < 5 \times 10^{-5}$). rs198712 showed the strongest association ($p = 5.4 \times 10^{-6}$) with odds ratios in males and females of 1.63 and 0.69 respectively. In the replication, interaction of sex with rs198712 was verified with $p = 0.022$.

Practical implications: The data provide evidence of a sexually dimorphic role of alleles at *NPY* in humans and support previous genome-wide findings of a role of *NPY* in PD.